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ORIGINAL PAPER

A hybrid swarm population of *Pinus densiflora* × *P. sylvestris* inferred from sequence analysis of chloroplast DNA and morphological characters

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Abstract: To confirm a hybrid swarm population of *Pinus densiflora* × *P. sylvestris* in Jilin, China, we used needles and seeds from *P. densiflora*, *P. sylvestris*, and *P. densiflora* × *P. sylvestris* collected from natural stands or experimental stations to study whether shoot apex morphology of 4-year old seedlings can be correlated with the sequence of a chloroplast DNA simple sequence repeat marker (cpDNA SSRs). Total genomic DNA was extracted and subjected to sequence analysis of the pine cpDNA SSR marker Pt15169. Results show that morphological characters from 4-year old seedlings did not correlate with sequence variants of this marker. Marker haplotypes from all *P. sylvestris* trees had a CTAT element that was absent from all sampled *P. densiflora* trees. However, both haplotype classes involving this insertion/deletion element were found in a *P. densiflora* × *P. sylvestris* population and its seedling progeny. It was concluded that the *P. densiflora* × *P. sylvestris* accessions sampled from Jilin, China resulted from bi-directional crosses, as evidenced by both species' cpDNA haplotypes within the hybrid swarm population.

Key words: *P. sylvestris* var. *sylvestriformis*; chloroplast DNA; simple sequence repeat sequencing; hybrid swarm population

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Introduction

The genus *Pinus* includes approximately 100 species and is one of the most widely distributed genera in the Northern Hemisphere (Farjon 2001). *Pinus sylvestris* is the most common pine from the Russian Far East to European countries. *Pinus densiflora* is native and distributed in China, Korea, and Japan. *Pinus sylvestris* var. *sylvestriformis* occurs in a very confined area near Baihe, Jilin Province, China, and was recognized as a variety in 1978 (Fu et al. 1999). The ranges of *P. densiflora* and *P. sylvestris* may not overlap where *P. densiflora* × *P. sylvestris* grows (Mirov 1967, refer to Fig. 3-50), although *P. densiflora* extended to the Jilin area where *Pinus sylvestris* var. *sylvestriformis* were grown at about 800 m elevation around Mt. Changbai (Szmidt and Wang 1993, refer to Fig. 1).

Pinus sylvestris var. *sylvestriformis* was clustered together with *P. densiflora* based on RAPD polymorphisms and chloroplast DNA simple sequence repeats (cpDNA SSRs). Based on RAPD marker analysis and from cpDNA microsatellite length and sequence analysis (Joung and Roh 2005), it was concluded to use of a hybrid formula, *P. densiflora* × *P. sylvestris*, rather than using an infraspecific taxon name based on either of the

parental species following the International Code of Botanical Nomenclature (Greuter et al. 2000). Natural hybridization in sympatric populations of different species of pines – *Pinus sylvestris* L., *P. mugo* Turra and *P. uliginosa* Nermann – has also been reported (Wachowiak and Prus-Glowacki 2008).

Szmidt and Wang (1993) suggested that interpopulation differentiation was higher in *P. densiflora* than *P. sylvestris* based on the intraspecific cpDNA variation. This might indicate that the divergence of *P. sylvestris* varieties occurred later than that among *P. densiflora* populations. An extensive survey for Chinese and Korean *P. densiflora* was considered important in order to better understand the evolutionary processes and genetic make-up of this species. Although some morphological differences in mature *P. densiflora* × *P. sylvestris* plants have been observed, this was not considered in sampling for the previous studies (Szmidt and Wang 1993; Joung and Roh 2005). Morphological characteristics of some *P. densiflora* × *P. sylvestris* individuals were very similar to those often observed in *P. densiflora* in Korea, and some were very similar to those observed in *P. sylvestris* in Germany (personal observation 2004).

The size of the *P. densiflora* × *P. sylvestris* population was estimated at 86,000 trees in Jilin, China, which are scattered in the area between 42°06′–42°32′ N and 128°07′–128°21′ E, but mostly concentrated around the Mei Ren Song Yuan, Jilin, China. It is not certain whether the current population of *P. densiflora* × *P. sylvestris* could be considered one population, resulting from the progeny of hybrids between *P. densiflora* and *P. sylvestris*, or a population of hybrid origin resulting from introcross of hybrids and their progeny as well as continued gene flow (backcrossing) from each parental species. Hybridization and introgression of genes could lead to evolution and speciation (Soltis and Soltis 2009). There have been no population studies on morphological patterns or their genetic correlations in *P. densiflora* × *P. sylvestris* populations.

Several cpDNA SSR markers were reported to resolve populations in *P. resinosa* with little morphological variations (Echt et al. 1998). Based on previous work by Joung and Roh (2005), the cpDNA SSRs marker Pt15169 and Pt 32024 (Vendramin et al. 1996) was selected from 11 SSR loci, and used to study the genetic differences between plants from two parental species and their progeny. In gymnosperms, cpDNA is paternally transmitted while mitochondrial DNA (mtDNA) is maternally inherited (Wagner 1992). The analysis of length polymorphisms of cpDNA SSRs repeat regions in pines may be considered suitable method for studying cytoplasmic genome inheritance and monitoring gene flow (Powell et al. 1996), which was also used in the previous work with nucleotide sequence analysis (Joung and Roh 2005).

The objectives of this study were: (a) to compare sequence data from a cpDNA SSRs marker of mother plants and seedling progenies for accessions of *P. densiflora* × *P. sylvestris* of different ages growing around Mt. Changbai, Jilin, PR China, and (b) to correlate cpDNA SSR marker sequence data with morphological characters observed at the shoot apex of 4-year old seedlings of *P. densiflora*, *P. sylvestris*, and *P. densiflora* × *P. sylvestris*.

Materials and methods

Plant materials

Seeds of *Pinus densiflora* × *P. sylvestris* from Jilin Province, PR China, of *P. densiflora* from Korea, Japan and Germany, and of *P. sylvestris* from Germany and a commercial source were used in this study (Table 1, Fig. 1). Mature plants of *P. densiflora* × *P. sylvestris* were selected from Mei Ren Song Yuan (accession nos. 1 through 13) and adjacent areas in Erdobaihe, Jilin. The morphology of accessions 1, 2, and 3 resembled that of *P. densiflora* (Joung and Roh 2005, refer to Fig. 1), and they were estimated to be about 300 years old. The morphology of accessions 4 through 13 could not be characterized whether they are *P. densiflora* or *P. sylvestris* type.

Seeds were sown in 15 cm pot filled with soilless medium and transplanted singly in pots (5 × 5 × 15 cm band) filled with Metro Mix 510 (Sun Grow Horticulture, Vancouver, British Columbia, Canada) in November, 2004. Slow release fertilizer, 14N-6.5P-10.8K Osmocote, was applied at 0.9 g per band at transplanting and spring of the following two years. Needles were picked from mother trees at the time of seeds collection from the natural sites and their progeny in 2007.

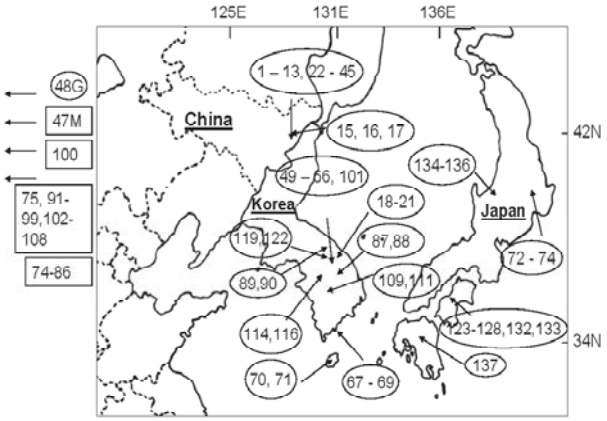

Fig. 1 Site information where accessions were collected. Accessions collected from the site outside China, Korea, and Japan are indicated with arrow sign. Accession numbers in circle were collected from the native sites. Refer to Table 1 for more detail.

Simple sequence repeats (SSR) analysis

In previous work (Joung and Roh 2005), the pine cpDNA SSR marker Pt15169 effectively differentiated the sequences of *P. densiflora* and *P. sylvestris*. DNA was extracted from selected needles using the CTAB method of Doyle and Doyle (1987). The cpDNA SSR regions were amplified using the Pt15169-F and -R primer set as described (Joung and Roh 2005) and the PCR products were direct sequenced. Partial sequence chromatogram of *P. densiflora*, accession no. 18, *P. sylvestris*, accession no. 91, and *P. densiflora* × *P. sylvestris* showing *P. sylvestris*

sequence, accession no. 1-A, and showing *P. densiflora* sequence accession no. 1-E is presented (Fig. 2)

Table 1. Accession data for *Pinus densiflora, P. sylvestris*, and *P. densiflora* × *P. sylvestris*

Acc. No. a	Description	Needle	Seed
1	*P. densiflora* × *P. sylvestris*, No. 23, 300 yr old, *P. densiflora* type [b]	X	X [c]
2	*P. densiflora* × *P. sylvestris*, No. 128, 300 yr old, *P. densiflora* type	X	X
3	*P. densiflora* × *P. sylvestris*, No. 140, 300 yr old, *P. densiflora* type	X	X
4–8	*P. densiflora* × *P. sylvestris*, small 1 - 5, ca.15 – 20 yr old	X	X
9–13	*P. densiflora* × *P. sylvestris*, medium 1/140, ca. 100 yr old	X	X
14	*P. densiflora*, Quarryhill Bot. Garden. Honshu, Japan	-	X
15	*P. densiflora* var. *sylvestriformis*, Yanbian, Jilin, China	X	-
16 - 17	*P. densiflora*, Yanbian, Jilin, China, 1999, SS Jeng	X	-
18	*P. densiflora*, Kangreung, Kangwon, Korea, JT Suh	X	X
19	*P. densiflora*, Daekwanryung, Korea, JT Suh	X	X
20	*P. densiflora*, Chungsun, Korea, KY Byun	X	X
21	*P. densiflora*, Plant Genetic Resources of Canada, collected from Japan	-	X
22–45	*P. densiflora* × *P. sylvestris*, Jilin, China, D. Mu, estimated about 100 – 300 yr old	X	X
46	*P. densiflora*, US National Arboretum (NA), NA 61727		X
47	*P. sylvestris* var. *mongolica*, NA 67306	X	-
47–1	*P. sylvestris*, PI 491519, Ames, Iowa, USA	X	-
48	*P. densiflora*, Germany, R. Röber,		X
49, 51, 52	*P. densiflora*, Angang 1, Kyungpuk-Do, Korea	X	-
50, 53	*P. densiflora*, Angang 2, Kyungpuk-Do, Korea	X	X
54, 59, 60	*P. densiflora*, Uljin 1, Kyungpuk-Do, Korea	-	X
55–58	*P. densiflora*, Uljin 2, Kyungpuk-Do, Korea	X	X
61	*P. densiflora*, Taebaek-san, Kangwon-Do, Korea, 1602-0523	X	-
62, 64	*P. densiflora*, Yeungnam Univ., Kyungpuk-Do, Korea	X	-
63, **65**	*P. densiflora*, Gyeongsan, Kyungpuk, Korea	X	X
66	*P. densiflora*, Daejeon, Korea, JS Lee.	X	X
67, 68, 69	*P. densiflora*, Wando, Cheonnam-Do, Korea, JO Park	X	
70, 71	*P. densiflora*, Kwaneum-sa, Jeju, Korea	X	X
72–74	*P. densiflora*, Tsukuba, Ibaraki, Japan	-	X
75	*P. sylvestris*, 85120 Schuarzwald Montane, Liliental, Liefernsamen Planaze, Germanay		X
77	*P. sylvestris*, Belgium, Schumacher Co., USA	-	X
78	*P. sylvestris*, Casadeen Massif, Schumacher Co., USA	-	X
79	*P. sylvestris*, Central Massif, Schumacher Co., USA	-	X
80	*P. sylvestris*, Gevaudan, Schumacher Co., USA	-	X
81	*P. sylvestris*, Guadarrama, Schumacher Co., USA	-	X
82	*P. sylvestris* var. *mongolica*, Schumacher Co., USA	-	X
83	*P. sylvestris*, Rhodopaea, Schumacher Co., USA	-	X
84	*P. sylvestris*, Riga, Schumacher Co., USA	-	X
85	*P. sylvestris*, Scotland, Schumacher Co., USA.	-	X
86	*P. sylvestris*, Turkey, Schumacher Co., USA	-	X
87, 88	*P. densiflora*, Andong, Kyungpuk-Do,Korea, JH Joung	-	X
89, 90	*P. densiflora*, Pyungchang, Kangwon-Do, Korea, KY Byun	-	X
91 – 99,	*P. sylvestris*, 305-85A, 497-25A, 436-57A, 1530-83C, 225-35D, 16536C, 11373B, 524-88B, 521-88A, Forest Research Center, Theisendorf, Germany	X	-
100	*Pinus sylvestris* var. *mongolica*, Wulranbatareu	X	-
101	*P. densiflora*, Taebaek-San, 1169-1977, Kangwon-Do, Korea	X	-
102–104	*P. sylvestris*, Forest Research Center, Theisendorf, Germany	X	-
105–108	*P. sylvestris*, accession number 594, 603, 613, 619, respectively,,Forest Research Station, Friburg, Germany	X	-
109, 111	*P. densiflora*, Ilsongjung, Daejeon , Korea	X	-
114, 116	*P. densiflora*, Mt. Geumsoo-san, Jechun, Chungbook-Do, Korea	X	-
119, 122	*P. densiflora*, Ducksan-ki, Jungsun, Kangwon-Do, Korea	X	-
123–125	*P. densiflora*, Niihama, Ehime, Japan	X	-
126, 128	*P. densiflora*, A1 Hwy 47, toward Mt. Akaishi, Matsuyama, Japan	X	-
129–131	*P. densiflora*, Tsukuba Botanical Garden, Tsukuba, Ibaraki, Japan	X	-
132–133	*P. densiflora*, Mt. Nishii, Matsuyama, Japan	X	-
134–136	*P. densiflora*, Kobujizawa, Nagano, Japan	X	-
137	*P. densiflora*, Kurume, Kyushu, Japan	X	-

[a] Germplasm was collected from the various botanical gardens and arboreta or purchased from a commercial company. If no other information is presented, germplasm was collected by M.S. Roh in collaboration with co-authors; [b] Canopy arrangement of No. 23 (accession 1), No. 128 (accession 2), and No. 140 (accession 3) (Mei Ren Song Yuan, Jilin, PR China) was similar to *P. densiflora* (Joung and Roh, 2005, refer to Fig. 1). The age of the tree was not verified, but estimated by counting the number of nodes where branches were formed; [c] X = Needles of mother trees and of seedlings obtained from their seed were analyzed.

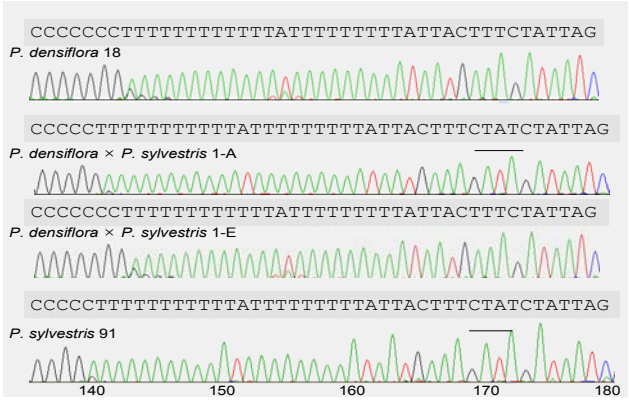

Fig. 2 Partial sequence chromatogram of *P. densiflora*, accession no. 18, *P. sylvestris*, accession no. 91, and *P. densiflora* × *P. sylvestris* showing *P. sylvestris* sequence, accession no. 1-A, and showing *P. densiflora* sequence accession no. 1-E. CTAT sequence presented on *P. sylvestris* cpDNA is indicated (underlined). Refer to Table 4 for accession information and comparison between morphological characteristics and sequence information.

Morphological characteristics

Dormant shoot apices of a *P. densiflora* seedling (Accession 109) and a *P. sylvestris* seedling (Accession 102) were photographed in December, 2008 (Fig. 3). Morphological characters of the vegetative buds and light green needles of *P. sylvestris* type and with white trichomes or hairs with the *P. densiflora* type with twisted dark green needles were used to assign seedlings of *P. densiflora* × *P. sylvestris* as either the *P. densiflora* or *P. sylvestris* morphotype. The shoot apex and needles shape and colors of all seedlings were examined during March – April, 2009, and assigned either as the *P. densiflora* or *P. sylvestris* type. This data was compared to the SSR sequence data discussed below. Also, 9 or 10 seeds of 15 *P. densiflora* × *P. sylvestris* accessions and one *P. densiflora* accession were scanned, and the length and width of each seed and of the wing of each seed were measured using a digimatic caliper. Data was subjected to analysis of variance, and means were compared by the honestly significant difference test (HSD) at the $p = 0.01$ level.

Results

Overall sequence analysis at the locus Pt15169 for *P. sylvestris* yielded a repeat of poly C -poly T1 -poly T2 followed by ATTACTTT*CTAT*; for *P. densiflora* it yielded poly C-poly T1-poly T2 followed by ATTACTTT ----, where *CTAT* was absent in *P. densiflora* (Fig. 2). The number of repeated single nucleotide in poly C, poly T1, and poly T2 regions in the locus Pt15169 for all accessions either from needles or seeds and also *P. densiflora, P. sylvestris*, and *P. densiflora* × *P. sylvestris* seedlings varies from 8 to 13. However, the majority in Poly C and Poly T2 regions showed 10 to 11 repeats and 9 repeats, respectively, in *P. densiflora* x *P. sylvestris* seedlings (Table 2, 3). There was no variation in the base sequence for a given taxon regardless of the collection sites where the accessions were obtained. This sequence pattern was observed in all *P. densiflora* and *P. sylvestris* accessions except for *P. densiflora* accession 74E, which showed the *P. sylvestris* type sequence (Table 4).

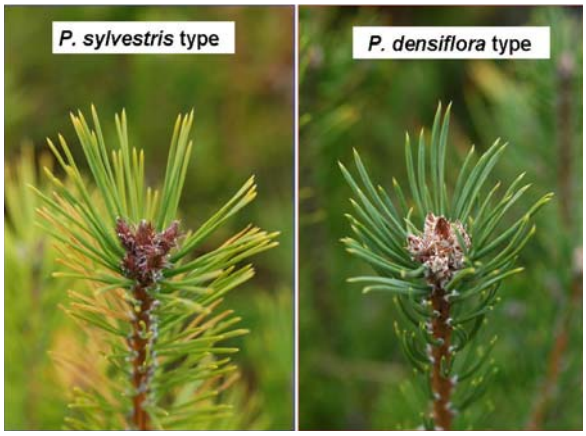

Fig. 3 Needle shape and vegetative shoot apex of 4-year old seedlings showing vegetative buds of *P. sylvestris* type with light green needles and with white trichomes (left) or hairs with the *P. densiflora* type with twisted dark green needles (right)

Table 2. Repeat of SSR sequence at locus Pt 15169 with variable sequence in intra species (small letter) and gaps (-) that indicate variations between aligned sequences of *P. densiflora*, *P. sylvestris*, and *P. densiflora* × *P. sylvestris*. The number of repeats in poly C, Poly T1 and Poly T2 are summarized in Table 3.

Taxon	Chloroplast DNA simple sequence repeats (cpDNA SSRs) at locus Pt15169			
	Poly C	Poly T1	Poly T2	CTAT
P. densiflora	CCCCCCCCccccc	TTTTTTTTttttt	TTTTTTTTtt	ATTACTTT----
P. sylvestris	CCCCCCCCCccc	TTTTTTTTTttt	TTTTTTTTTt	ATTACTTTCTAT
P. densiflora × *P. sylvestris*	CCCCCCCCCccc	TTTTTTTTTtttt	TTTTTTTTTt	ATTACTTTctat

Table 3. Repeat of SSR sequence at locus Pt 15169 with variable sequences. The number of repeats in Poly C, Poly T1, and Poly T2, which varies from 8 to 13, and the percentage of *P. densiflora* (*P. den*), *P. sylvestris* (*P. syl*), and *P. den* × *P. syl.*

No. of repeat	Repeat of nucleotide [a]								
	Poly C			Poly T1			Poly T2		
	P. den × *P. syl*	*P. den*	*P. syl*	*P. den* × *P. syl*	*P. den*	*P. syl*	*P. den* × *P. syl*	*P. den*	*P. syl*
8	0	0	0	0	0.6	2.3	0	1.3	0
9	0.8	4.5	17.5	17.5	0.6	11.4	84.5	67.5	97.7
10	61.8	61.4	12.0	12.0	5.1	38.6	15.5	31.2	2.3
11	31.1	27.3	43.4	43.4	54.8	40.9	0	0	0
12	6.4	6.8	18.3	18.3	29.3	6.8	0	0	0
13	0	0	8.8	8.8	9.6	0	0	0	0

[a] Sequence analysis for all mother *P. syl* trees yielded poly C-poly T1-poly T2- ATTACTTT*CTAT* and for all mother *P. den* trees yielded poly Cc -poly T1-poly T2- ATTACTTT----.

However, in *P. densiflora* × *P. sylvestris* accessions, comparing sequence data between mother trees and their seedling progeny revealed that they did not have the same sequence (Table 4). For example, in accession 1, the mother tree yielded the *P. densiflora* type sequence with the absence of CTAT. However, 3 out of 12 seedlings (1E, 1G, and 1I) showed the *P. densiflora* type sequence, while the rest showed the *P. sylvestris* type sequence. In accession 2, the mother trees yielded *P. sylvestris* type sequence with CTAT, while 4 out of 12 seedlings (2A, 2D, 2I, 2K) showed the *P. densiflora* type sequence. This variation was observed regardless of the age of mother plants, except accessions 4, 8, and 9 where all seedlings showed the *P. sylvestris* type (Table 4).

Table 4. Overall sequence analysis for *P. densiflora, P. sylvestris*, and *P. densiflora* × *P. sylvestris* using Pt15169 cpDNA SSR primer set.

Taxon	Accession number [a]	*P. densiflora* type			*P. sylvestris* type		
		M [b]	Seedling Numbers		M	Seedling Numbers	
			Morphology [c]	cpDNA SSRs		Morphology	cpDNA SSRs
P. densiflora × *P. sylvestris* [d]	1	X	A, B, D, F, H, I, K, L [e]	E, G, I		C, E, G, J	A, B, C, D, F, H, J, K, L
	2		B, C, D, E, F, G, H, J, K, L	A, D, I, K	X	A, I	B, C, E, F, G, H, J, L
	3		A, B, E, F, G, H, I, J, L	C, J	X	C, D, K	A, B, D, E, F, G, H, I, K, L
	4	X	A, B, C, E, F			D	A, B, C, D, E, F
	5		A, D, E, F	D	X	B, C	A, B, C, E, F
	6	X	A, C, H	C, D		B, D, F	A, B, E, F
	7	X	A, F	E, F		B, C, D, E	A, B, C, D
	8		A, B, C, F		X	D, E	A, B, C, D, E, F
	9		B, D, E,		X	A, C, F	A, B, C, D, E, F
	10		A, B, D, E	A, D, E	X	C, F	B, C, F
	11			- [f]	X		-
	12		A, D, E, F	D	X	B, C	A, B, C, E, F
	13		A, C, F	A, D, E	X	B, D, E	B, C, F
P. densiflora × *P. sylvestris*	15			-	X		-
	22	X	A, C, E	A		B, D, F	B, C, D, E, F
	23		B, D, E	A, D, E, F	X	A, C, F	B, C
	24		C, D, F	E, F	X	A, B, E	A, B, C, D
	25 (1) [g]				X	B	B
	26		A, C, D,	B, D	X	B, E, F	A, C, E, F
	27	X	B, C, E, F	A, B, F		A, B	C, D, E
	28	X	A, D, F	A, B, E		B, D, E	C, D, F
	29	X	A, B, D, E	B, E, F		C, F	A, C, D
	30		C, D, F	C, D. E	X	A, B, E	A, B, F
	31		A, C, D, F	A, E, F	X	B, E	B, C, D
	32		A, E, F		X	B, C, D,	A, B, C, D, E, F
	33		B, C, F	C, D	X	A, D, E	A, B, E, F
	34		A, C, D,		X	B, E, F	A, B, C, D, E, F
	35	X	A, B, E, F	B, C, E, F		C, D	A, D
	36		C, D, E, F	D	X	A, B	A, B, C, E, F
	37		D, E, F		X	A, B, C	A, B, C, D, E, F
	38	X		-			-
	39 (1)		A	A	X		-
	40 (5)		A, B, D	A, C	X	C, E	B, D, E
	41		B, C, E,	E	-	A, D, F	A, B, C, D, F
	42 (3)		A, B,		X	C	A. B, C
	43	X	C, D, E,	C, D, E, F		A, B, F	A, B
	44		A, C, E, F	A, C, E, F	X	B, D	B, D
	45		A, C, D,	C, D, E, F	X	B, E, F	A, B
P. densiflora	18 – 20, 53, 55 (5), 58	X	A, B, C, D, E, F	A, B, C, D, E, F			
	21		A, B, C, D, E, F	A, B, C, D, E, F			
	50	X	A, B, C, D, E, F	A, B, C, D, E, F			
	51						
	52						
	61 (1)			A			
	62 (4), 63 (1), 65 (4), 66 (1)	X		A, B, C, D			
	67 – 69	X					
	70, 72, 75, 87 - 90	X		A, B, C, D, E, F			
	71 (4)	X		A, B, C, D			
	73 (3)	X		A, B, C			
	74	X		E			A, B, C, D, F

Continued Table 4

Taxon	Accession number [a]	*P. densiflora* type			*P. sylvestris* type		
		M [b]	Seedling Numbers		M	Seedling Numbers	
			Morphology [c]	cpDNA SSRs		Morphology	cpDNA SSRs
	96–99, 109, 111, 114, 116, 119, 122, 123, 124–126, 128, 129, 131, 132, 133–137	X	All				
P. sylvestris	76, 77, 83, 84 (4), 86 91 – 94, 102-108				X	A, B, C, D, E, F	A, B, C, D, E, F All
P. sylvestris var. *mongolica*	100				X		-

[a] Refer to Table 1 for accession information; [b] M, needles collected from the mother plants, and indicated with X based on the sequence analysis data, i. e., accession 1 gave *P. densiflora* type data and accession 2 and 3 gave *P. sylvestris type* data; [c] Morphology of dormant shoot apex. Refer to Fig. 3; [d] *Pinus sylvestris* or *P. densiflora* type based on data from needles of mother trees; [e] Samples from 12 seedlings were sequenced for accessions 1, 2, and 3, and from six seedlings for all other accessions, unless otherwise indicated; [f] -: No sample was analyzed; [g] The number in the parentheses indicates the number of seedlings sequenced. Some seedlings did not yield good DNA or less than 6 seeds were obtained.

Morphological characters of the dormant shoot apex of *P. densiflora* and *P. sylvestris* seedlings (Fig. 3) did not show any significant variation among seedlings in either taxon (Table 4). However, dormant shoot apices of *P. densiflora* × *P. sylvestris* seedlings showed variation, which was not congruent with cpDNA SSRs sequence data. For example, morphological characters of mother plants of *P. densiflora* × *P. sylvestris* accessions 1, 2, and 3 were typical of the *P. densiflora* type. However, their cpDNA SSRs sequences showed the *P. densiflora* type for accession 1 and the *P. sylvestris* type for accessions 2 and 3.

There was a significant difference in the wing and seed size among *P. densiflora* × *P. sylvestris* accessions (Table 5, Fig. 4). The length of the wing of seeds was greatest with accession 8 (1.82 cm), which was significantly longer than that of most accessions except with accessions 9 and 24 and the width with accession 12 (0.77 cm) except with accession 3, and which are significantly longer from those of *P. densiflora* and those of *P. sylvestris*. Although the color, shape, and streaking pattern shown in seed wings of *P. densiflora* × *P. sylvestris* did not differ greatly within each accession (images not presented), they differed greatly between accessions.

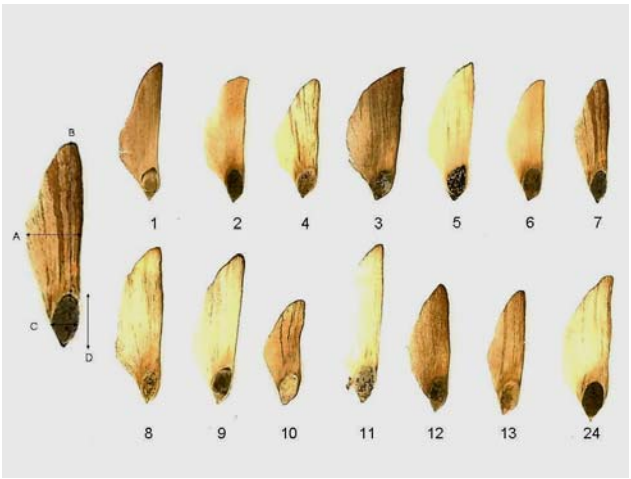

Fig. 4 Shape and size of individual seeds of *P. densiflora* × *P. sylvestris* collected from Baihe, Jilin, China. Refer to Table 1 for accession information.

Table 5. The size of wing and seeds of selected *P. densiflora* × *P. sylvestris* and *P. densiflora.*

Accession information [a]	Wing		Seed	
	Width (A) [b] (cm)	Length (B) (cm)	Width (C) (cm)	Length (D) (cm)
P. densiflora × *P. Sylvestris* 1	0.49	1.34	0.221	0.397
2	0.52	1.43	0.200	0.468
3	0.72	1.57	0.268	0.463
4	0.66	1.36	0.298	0.425
5	0.53	1.59	0.244	0.500
6	0.54	1.37	0.242	0.467
7	0.59	1.45	0.315	0.490
8	0.61	1.82	0.247	0.401
9	0.57	1.74	0.323	0.449
10	0.61	1.11	0.302	0.514
11	0.57	1.67	0.269	0.521
12	0.77	1.47	0.277	0.479
13	0.51	1.37	0.258	0.458
14	0.78	1.67	0.302	0.534
34	0.57	1.11	0.294	0.514
P. densiflora 51	0.59	1.38	0.315	0.438
Level of significance at 1% [c]	0.093	0.198	0.0157	0.0218

[a] Refer to Table 1 for details on collection information. *Pinus sylvestris* seeds with intact wing was not available; [b] Refer to Fig. 4, insert for measurement; [c] Honestly significant difference, F-test.

Discussion

Pt 15169 cpDNA SSR marker analysis effectively distinguished *P. densiflora* from *P. sylvestris*, and the results agree with the previous finding that *P. densiflora* × *P. sylvestris* is of hybrid origin, and should not be considered as *P. sylvestris* var. *sylvestriformis* – a lower rank of *P. sylvestris* (Joung and Roh 2005). Although the majority of plants collected as *P. densiflora* × *P. sylvestris* could be a hybrid origin, it may be possible that some of those collected as *P. densiflora* × *P. sylvestris* can be considered as *P. densiflora* (e. g. accession 28) or as *P. sylvestris* (e. g. accessions 4, 8, 9, 32, 34, and 37) since the mother tree and all of

its seedlings showed the same cpDNA SSR sequence as the referred taxon.

However, the discrepancies between morphological characters and cpDNA SSRs sequence of most of *P. densiflora* × *P. sylvestris* accessions confirms their hybrid origin based on both morphological characters and the cpDNA SSRs sequence. Although the seedling sample size for individual accessions, ranging from 6 to 12 per accession, could be of concern, the number of samples from all accessions is large enough to give confidence in the results. The observations from many accessions confirm the hybrid origin of *P. densiflora* × *P. sylvestris* and the existence of hybrid swarm along with two parental taxa at the native sites.

The pattern of inheritance of cpDNA SSR genes observed in younger plants (accessions Nos. 4–8 and 10–13) were comparable to those observed in older plants (accessions 1 to 3), which may suggest that the seeds formed resulted from outcrossing, as also reported for *P. densiflora* (Lian et al. 2001). The source of pollen is likely from the same population of *P. densiflora* × *P. sylvestris* with a mixed cpDNA genotype of *P. densiflora* and *P. sylvestris* since the distance among accession Nos. 1 through 13 was less than 60 m, and no *P. densiflora* and *P. sylvestris* plants grow sympatric. The average distance of pollen migration for a small population of *P. sylvestris* within the stand was reported to be 48 m or 83 m excluding selfs (Robledo-Arnuncio and L. Gil 2005). Since cpDNA is paternally inherited in *Pinus*, the segregation ratio to *P. densiflora* and *P. sylvestris* type would theoretically and eventually be 1: 1, if the *P. densiflora* × *P. sylvestris* population results from the same mating system as the parents and seed production responds to the mixed pollen pools. In both the old plants (accessions 1, 2 and 3) and young plants (accessions 4–8), the ratio is far from the theoretical ratio of 1:1. This is expected since in a pine hybrid swarm population progeny especially originating from bidirectional crosses are not expected to have the same cpDNA haplotypes as their mother due to the pollen cloud population is a mixture of different cpDNA haplotypes, so seed progeny cpDNA will reflect that mixture.

No study has been carried out comparing the pollen viability of *P. densiflora* × *P. sylvestris* individuals that show either the *P. densiflora* type or *P. sylvestris* type cpDNA SSRs sequences, or the competitiveness of pollen of the two parental taxa. Nevertheless, it is likely that mixed pollen from *P. densiflora* × *P. sylvestris* and the two parental taxa, particularly from *P. densiflora* which may grow in sympatric could contribute to differences in paternally inherited cpDNA SSRs genes in the progenies. This would be similar to the polycrosses reported in *Pinus radiata* D. Don (Moran and Griffin 1985). The presence of both *P. densiflora* and *P. sylvestris* genes in seedlings of *P. densiflora* × *P. sylvestris* suggests that they are reverting to one of the sympatric parents and do not exist as a stabilized introgressant (Szmidt and Wang 1993). Pollen from *P. sylvestris* or possibly *P. densiflora* may not be available since *P. sylvestris* is reported not grow sympatric with *P. densiflora* (Mirov 1967) and *P. densiflora* × *P. sylvestris*. *Pinus densiflora* was not observed at northwestern part of Mt. Changbai, China, although it is noted to grow sympatric with *P. densiflora* × *P. sylvestris* (Szmidt and Wang 1993). *Pinus densiflora* is growing in most of northern part of Korea near Mt. Backdoo (Mt. Changbai) at the elevation of lower than 1,000 m above the sea level (Lim 1996). Around the area where *P. densiflora* × *P. sylvestris* is growing, the presence of *P. densiflora* was not observed (Roh, personal observation). The possibility that pollen from *P. densiflora* could contribute the hybridization may not be excluded. The discrepancies on the boundary of *P. densiflora* distributed in the northern part of Korea and Jilin, China (Lim 1996; Mirov 1967; Szmidt and Wang 1993) should further investigated, since *P. densiflora* was observed at Mt. Myohyang (40.03° N, 126.28° E) (Roh, personal observation 2001).

Based on the morphology and the cpDNA SSRs markers, the percentages of the estimated 86,000 trees *P. densiflora* × *P. sylvestris* population that may be considered as either one of the parental species, *P. densiflora* or *P. sylvestris*, cannot be determined. In a sympatric population of *Pinus taeda* L. and *P. echinata* Mill, 2 and 8 hybrids, respectively, among 80 mature plants, including post F_1 hybrids, had morphological characters that were congruent with microsatellite data, and some hybrids likely resulted from early-generation introgression from either one of the parents (Chen et al. 2004). A similar situation was also reported in the hybrid zone between *P. sibirica* Du Tour and *P. pumila* (Pall.) Regel (Petrova et al. 2008). Viability of pollen of interspecific hybrids of *P. densiflora* × *P. sylvestris* has not been examined at present. However, it may be expected to be low compared to that of its parental taxa based on findings of high percentage of sterile pollen in hybrid swarm populations of *Pinus mugo* and *P. sylvestris* (Kormutak et al. 2007).

Understanding the degree of pollen viability, pollen compositions, and seed set in *P. densiflora* × *P. sylvestris* in relation to the inheritance pattern of cpDNA of the progeny would be useful in understanding the variation in the DNA sequence data for the locus investigated, and also its relationship to the morphological characters observed in seedling progeny and later in mature trees whether *P. densiflora* × *P. sylvestris* population. All of this information should be investigated to understand pollination patterns and inheritance or segregation in *P. densiflora* × *P. sylvestris* populations. Controlled pollinations among hybrids of *P. densiflora* × *P. sylvestris* and back-crosses to both of the parents should be carried out to understand all of these issues.

Pinus densiflora × *P. sylvestris* was reported to have morphological characters intermediate to its two parental taxa (Cheng and Fu 1978), but the description lacks identification of individual plants in the wild (Joung and Roh 2005, refer to Fig. 1,). It would be difficult to describe intermediate morphological characters even in the hybrid populations resulting from introgressants from either parent to form a hybrid swarm. Bidirectional introgression resulting from backcrosses of early generation hybrids may be responsible for difficult to distinguish hybrids and its parental species. Only the SSRs data, not the morphological characteristics, identified the hybrids or introgressants in interspecific hybrids between *Pinus taeda* L. and *P. echinata* Mill. (Chen et al. 2004).

Morphological characters of the shoot apex from four-year old *P. densiflora* × *P. sylvestris* seedlings were classified as being either the *P. densiflora* or *P. sylvestris* type. However, these characters were not correlated with the respective cpDNA SSRs

sequence data. Correlation of the identification using molecular markers and morphological characters have successfully characterized hybrid origin in *Arisaema* (Lee et al. 2011), *Pulsatilla* (Lee et al. 2010), *Clematis* (Yuan et al. 2010), and *Cyrtanthus* (Lee et al. 2012). Further, though the size of seeds and seed wings and streaking pattern observed in the seed wings does not differ in a given accession, these characters also did not correlate with cpDNA SSRs sequence data. When all seedlings from accessions 1 through 45 were considered, a ratio of 68:146 (0.466:1) in cpDNA SSR sequence data and 128:86 (1.488:1) in morphological characters for the *P. densiflora*:*P. sylvestris* type was obtained. Height of seedlings can possibly be used to differentiate parental taxa, since generally *P. sylvestris* seedlings are >15 cm taller than *P. densiflora* when seedlings were grown under the same cultural conditions, *i. e*., pot size, nutrition regimes, and temperature and light intensity. When it was difficult to assign seedlings of *P. densiflora* × *P. sylvestris* to one of the parent taxa based on the shoot apex, height was used as a secondary criterion to assign a seedling to one of their parental taxa. It was concluded that size and shape of seeds and shoot apex morphology in seedling stages cannot be used to unambiguously predict the cpDNA haplotype of seedlings of *P. densiflora* × *P. sylvestris*.

Conclusion

Morphology of the shoot apex from 4-year old seedlings of *P. densiflora* × *P. sylvestris* is not correlated with cpDNA SSRs data, and can not be used to trace to either one of their parental taxa. The hybrid origin of *P. densiflora* × *P. sylvestris* and the swarm of hybrids population with their two parental taxa were clearly demonstrated from cpDNA SSRs data. To understand the segregation of traits in *P. densiflora* × *P. sylvestris*, progeny of two parental taxa and backcrosses of the hybrids back to either one of parents should be carried out utilizing populations obtained from controlled pollination.

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References

Chen J, Tauer CG, Bai B, Huang Y, Payton ME, Holley AG. 2004. Bidirectional introgression between *Pinus taeda* and *Pinus echinata*: evidence from morphological and molecular data. *Can J For Res*, **34**: 2508−2516.

Doyle JJ, Doyle, JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull*, **19**: 11−15.

Echt CS, DeVerno LL, Anaidei M, Vendramin GG. 1998. Chloroplast microsatellites reveal population genetic diversity in red pine, *Pinus resinosa* Ait. *Mol Ecol*, **7**: 307−316.

Farjon A. 2001. World checklist and bibliography of conifers. 2nd ed. Richmond, Surrey, U. K. Royal Bot Gard, p. 309.

Fu L, Li N, Mill RR. 1999. *Pinus* Linnaeus. In: *Flora of China, Vol. 4*. Beijing: Science Press and St. Louis, MO, USA: Missouri Bot Gard Press, pp. 12–25

Greuter W, McNeill J, Barrie FR, Burdet H-M, Demoulin V, Filgueras TS, Nocolson DH, Silva PC, Skog, Trehane P, Turland NJ, Hawksworth DL. 2001. International Code of Botanical Nomenclature (St. Louis Code). Regnum Vegetabile 138. Königstein: Koeltz Scientific Books,

Joung YH, Roh MS. 2005. Mapping characterization of *Pinus sylvestris* var. *sylvestriformis* based on chloroplast DNA microsatellite markers. *For Genet*, **12**: 89–98.

Kormutak A, Bohovičova J, Vookova B, Gömöry D. 2007. Pollen viability in hybrid swarm populations of *Pinus mugo* Turra and *P. sylvestrisvestris* L. *Acta Biologica Cracoviensia*, *Series Botanica*, **49/1**: 61−66.

Lee AK, Hong J, Bauchan GR, Park SH, Joung YH. 2012. Confirmation of hybrid origin of *Crytanthus* based on the sequence analysis of internal transcribed spacer. *Sci Horti*, **144**: 153−160.

Lee AK, Joung YH, Wu XW, Jung HK, John Burk C, Sanders, LL, Roh MS, Suh, JK. 2011. Confirmation of hybrid origin in *Arisaema* (Araceae) using molecular markers. *Sci Hortic*, **129**: 812−817.

Lee AK, Yuan T, Suh JK, Choi DS, Roh MS, Joung YH, Ko JY, Lee JS. 2010. *Pulsatilla tonghangensis*, a natural hybrid sarm population hybridized with *P. koreana* based on RAPD and SNPs of chloroplast NDA. *Hort Environ Biotechnol*, **54**: 409–421.

Lian C, Miwa M, Hogetsu T. 2001. Outcrossing and paternity analysis of *Pinus densiflora* (Japanese red pine) by microsatellite polymorphism. *Hered*, **87**: 88–98.

Lim RJ. 1996. Korean forest trees and shrubs (colour illustrations). Pyongyang, DPR Korea (in Korean): Industry Press Group, p. 426.

Moran GF, Griffin AR. 1985. Non-random contribution of pollen in polycrosses of *Pinus radiata* D. Don. *Silvae Genet*, **34**: 117−121.

Ohta A, Murakawa Y. 2002. Recessive inheritance of morphological characteristics of utsukushimatsu, *Pinus densiflora* f. *umbraculifera. J For Res,* **7**: 153−156.

Petrova EA, Goroshkevich SN, Belokon MM, Belokon YS, Politov DV. 2008. Population genetic structure and mating system in the hybrid zone between *P. sibirica* Du Tour and *P. pumila* (Pall.) Regel at the Eastern Baikal Lake Shore. *Ann For Res*, **51**: 19–30.

Powell W, Mogante M, McDevitt R, Vendramin GG, Rafalski JA. 1995. Polymorphic simple sequence repeat regions in chloroplast genomes: Applications to the population genetics of pines. *Proc Nat Acad Sci*, **92:** 7759−7763.

Robledo-Arnuncio JJ, Gil L. 2005. Patterns of pollen dispersal in a small population of *Pinus sylvestris* L. revealed by total-exclusion paternity analysis. *Hered*, **94**: 13−22.

Soltis PS, Soltis DE. 2009. The role of hybridization in plant speciation. *Ann Rev Plant Biol*, **60**: 561−588.

Szmidt AE, Wang XR. 1993. Molecular systematics and genetic differentiation of *Pinus sylvestris* (L.) and *P. densiflorasiflora* (Sieb. Et Zucc). *Theor Appl Genet*, **86**: 159−165.

Takenouchi M. 1942. A preliminary report on the conifers of Manchukuo. *J Japan For Soc*, **24**: 113−129.

Vendramin GG, Lellim L, Rossi P, Morgante M. 1966. A set of primers for amplification of 20 chloroplast microsatellite in Pinaceae. *Mol Ecol*, **5**: 595−598.

Wachowiak W, Prus-Glowacki W. 2008. Hybridization processes in sympatric population of pines *Pinus sylvestris* L., *P. mugo* Turra and *P. uliginosa* Nermann. *Plant Syst Evol*, **271**: 29–40.

Wagner DB. 1992. Nuclear, chloroplast, and mitochondrial DNA polymorphism as biochemical markers in population genetic analyses of forest trees. *New For*, 6: 373–390.

Yuan T, Wang IY, Roh MS. 2010. Confirmation of *Clematis* hybrids using molecular markers. *Sci Hortic*, **125**: 136−145.